ELSEVIER

Fish & Shellfish Immunology 20 (2006) 20–28

Fish & Shellfish Immunology

www.elsevier.com/locate/fsi

A vaccination and challenge model using calcein marked fish

Phillip H. Klesius [a,*], Joyce J. Evans [b], Craig A. Shoemaker [a], David J. Pasnik [b]

[a] *U.S. Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory, P.O. Box 952, Auburn, AL 36831-0952, USA*

[b] *U.S. Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory, 151 Dixon Drive, Chestertown, MD 21620, USA*

Received 19 August 2004; revised 31 January 2005; accepted 1 March 2005
Available online 27 April 2005

Abstract

A vaccination and challenge cohabitation model was established and evaluated using Nile tilapia (*Oreochromis niloticus*), the fluorescent chromophore calcein, and a *Streptococcus iniae* vaccine. Tilapia were non-invasively calcein marked, sham-vaccinated (CMSV) and cohabited with non-marked sham-vaccinated (NMSV) or non-marked *S. iniae* vaccinates (NMV) as a single unit. After 30 d, the cohabitants were challenged with a virulent isolate of *S. iniae* by intraperitoneal (ip) injection and the cumulative mortality was measured over a period of 15 d. Calcein marking did not have a significant effect on *S. iniae* susceptibility as mortality of CMSV and NMSV was not significantly different ($P = 0.6756$). Nor did calcein marking have an effect on the vaccination and challenge cohabitation model. The results showed that the cumulative mortality of CMSV ($N = 160$) was significantly greater ($P < 0.0003$) than those of NMV ($N = 160$). The results of the calcein marking trials indicate that the most suitable calcein concentration and exposure time to produce detectable fluorescent marking of tilapia was 500 mg L^{-1} for 4 h. Furthermore, the calcein marks were readily visible in the calcified skeletal structures of head and fins using a portable handheld UV lamp set at 365 nm wavelength. Calcein appears to be a valuable tool for non-invasive, non-lethal, non-stressful, mass marking of fish to differentiate between sham- and pathogen-vaccinated fish in this cohabitation model. The vaccination and challenge cohabitation model also offers the statistical advantage of using individual fish as the experimental unit maintained in the same aquarium.
Published by Elsevier Ltd.

Keywords: Vaccination; Cohabitation model; Calcein; Marking; Nile tilapia; *Streptococcus iniae*

* Corresponding author. Tel.: +1 334 887 3741.
E-mail address: pklesius@ars.usda.gov (P.H. Klesius).

1050-4648/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.fsi.2005.03.003

1. Introduction

Cohabitation, where groups of fish are held in the same rearing unit, is regarded as one of the best models for evaluation of vaccine potency because it most mimics natural conditions of pathogen transfer [1]. A number of techniques exist for marking fish for such trials. These techniques include fin clips [2], percutaneous tags [3], visible implant tags [4], and coded wire tags [5]. These techniques are generally unsuitable for marking large numbers of fish or small size fish. Fin clips, tags, electric, and freeze brands can also result in compromised protection of the body surface, stress, and behavioural effects. More importantly, these fish marking techniques may injure the cutaneous barrier against infection and pose an artificial port of entry for waterborne pathogens [6]. Thus, an alternative technique of marking fish needed to be investigated to successfully develop an experimental vaccination and challenge cohabitation model.

A non-invasive and non-lethal marking technique to differentiate between sham-vaccinated (control) and vaccinated cohabitants appeared to be the use of the fluorescent chromophore calcein [7]. Calcein has proven to be an effective chemical marker in several fish species, including: killifish, *Heterandria formosa* [8]; zebrafish, *Danio rerio* [9]; guppies, *Poecilia reticulata* [10,11]; striped bass, *Morone saxatilis* [12]; red drum, *Scianops ocellatus* [13]; Atlantic croaker, *Micropagonias undulatus*; spot, *Leiostomus xanthurus* [14]; spotted seatrout, *Cynoscion nebulosus* [15]; brook trout, *Salvelinus fontinalis* [16]; rainbow trout, *Oncorhynchus mykiss* [17]; silver perch, *Bairdiella chrysoura* [18]; summer flounder, *Paralichthys dentatus* [19]; and Atlantic salmon, *Salmo salar* [7,16,20]. However, no information was available on marking Nile tilapia (*Oreochromis niloticus*) with calcein.

Furthermore, although calcein marking techniques have been employed for a variety of reasons, including distinguishing hatchery reared fish from wild fish, growth rate, survival rate, and migrations of stocked fish, this marking technique has not yet been employed in a vaccination and challenge cohabitation model.

An alternative means of detecting calcein marking by use of a portable UV lamp that would allow for non-injurious detection of marking on both live and dead fish (in the lab or field) was also investigated. In order to develop and evaluate a vaccination and challenge cohabitation model, the first objective of this study was to evaluate calcein marking and detection to distinguish between calcein marked sham-vaccinates (CMSV) and non-marked sham-vaccinates (NMSV). A further objective was to evaluate calcein marking and detection to distinguish between CMSV controls and NM *S. iniae* vaccinated (NMV) cohabitants. A modified *Streptococcus iniae* vaccine [21,22] and challenge with a virulent *S. iniae* isolate was used to evaluate the vaccination and challenge cohabitation model. Intraperitoneal injection was chosen for the challenge of control and vaccinated fish, because this method is regarded as highly reproducible and a reliable method in the evaluation of vaccines [1].

2. Materials and methods

2.1. Fish

Nile tilapia (*O. niloticus*) were obtained from stocks maintained at ARS, USDA, Aquatic Animal Health Research Laboratory (Auburn, AL). Tilapia (mean weight of 15 ± 2 and 20 ± 2 g each) were acclimated in flow-through 57-L aquaria supplied with 0.5 L h^{-1} dechlorinated water for 10 d prior to experiments. A light:dark period of 12:12 h was maintained and aeration supplied by an air stone. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO[1]). To verify the *S. iniae*-free status of the fish, samples were obtained for bacterial culture by passing an inoculation loop into the brain and kidney of 20

[1] Use of trade or manufacturer name does not imply endorsement by the US Department of Agriculture.

fish sampled from the population of origin. The samples were streaked directly on sheep blood agar and incubated at 27 °C for 24–48 h. None of the fish sampled were found to be culture positive for *S. iniae*. The dissolved oxygen, temperature, pH, salinity, hardness, ammonia, and nitrites were measured using CEL/890 Advanced Portable Laboratory, Hach (Loveland, CO 80539). In all trials, the mean ± standard deviation of dissolved oxygen (mg L^{-1}) was 5.9 ± 0.56, temperature (°C) was 26.1 ± 0.63, pH was 7.9 ± 0.01 and hardness (mg L^{-1}) was 100 ± 10.0. Ammonia and nitrite concentrations (mg L^{-1}) were below the detection limits.

2.2. Bacteria

Streptococcus iniae isolate ARS #60 (isolated from hybrid striped bass, *Morone chrysops* × *M. saxatilis*, with natural streptococcal disease) was used in the preparation of the vaccine and for ip challenge. The isolate was identified as *S. iniae* by standard methods [23,24]. The isolate was grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, MD) for 24 h at 27 °C and then frozen in 2.0 mL aliquots at −80 °C.

2.3. Vaccine preparation

Vaccine was similarly prepared as previously described by Klesius et al. [21,22]. Briefly, *S. iniae* was stationary cultured in TSB for 96 h at 27 °C. Culture was treated at 27 °C for 24 h with 10% neutral buffered formalin to give a final concentration of 3%. The formalin-treated culture was centrifuged at 700 × g for 30 min and cell pellet and culture fluid separated. The cell free culture was concentrated 20-fold using a 2 kDa spiral concentrator, filtered sterilised (0.2 μm), and used to re-suspend the cell pellet in V/V of 10:1. The calculated final concentration of the vaccine was 4×10^9 CFU mL^{-1} (based on pre-incubation plate count) corresponding to 1.9 optical density at 540 nm. The vaccine was considered killed by lack of growth on sheep blood agar at 72 h at 27 °C. The vaccine was administered by ip injection in a volume of 100 μL and sham-vaccinated fish were ip injected with the same volume TSB. The *S. iniae* vaccine has been patented (US 6,379,671B1) by Klesius et al. [25].

2.4. Calcein marking, duration of exposure, percent survival, intensity, duration of fluorescent marking and detection techniques

The chemical used to mark fish was calcein (C_{30} H_{26} N_2 $O_{13,}$ Sigma Chemical Co., St Louis, MO). Bath immersion solutions were prepared by dissolving 250 or 500 mg L^{-1} of dechlorinated water (dissolved oxygen, mg L^{-1} was 3.5 ± 0.56, temperature (°C) was 26.1 ± 0.63 and pH was 7.9 ± 0.05). The pH of calcein solutions was not adjusted or buffered. Table 1 shows the changes in water quality characteristics of 500 mg L^{-1} calcein in 1 L bath solution following immersion of 10 tilapia for 4 h. The pH of the bath

Table 1
Hourly changes in water quality characteristics of 3 L of 500 mg L^{-1} calcein bath solution following immersion of ten 15 g tilapia for 4 h

Water quality	Exposure time (h)				
	0	1	2	3	4
Dissolved oxygen (mg L^{-1})	3.5 ± 0.56[a]	6.6 ± 0.12[b,c]	6.3 ± 0.12[b]	6.3 ± 0.14[b]	6.4 ± 0.17[b]
Temperature (°C)	26.1 ± 0.63[a]	24.9 ± 0.15[a,b]	24.5 ± 0.27[b,c]	24.4 ± 0.27[b,c]	24.4 ± 0.27[b,c]
pH	7.9 ± 0.05[a]	4.8 ± 0.82[b,c]	5.2 ± 0.18[b]	5.5 ± 0.78[b]	5.3 ± 0.48[b,c]

Data are means and standard deviations of the water quality characteristics in three replicated immersion baths. Different superscripts in each row indicate significant differences at the 95% level.

solutions was significantly decreased ($P < 0.05$) within 1 h. The dissolved oxygen levels significantly increased ($P < 0.05$) at 1 h. The water temperature significantly decreased ($P < 0.05$) after 2 h.

The CM tilapia (120) were bath immersed for 4, 24, or 48 h with aeration in funnel or cone lidded buckets containing 12 L of either 250 mg L^{-1} or 500 mg L^{-1} calcein solutions. Another 20 fish were immersed in water without addition of calcein (NM controls) (Table 2). After immersion, the fish were rinsed a number of times in fresh water, then placed in aquaria (20 fish/aquarium) with flow-through water for 16–18 h to remove any excess or unbound calcein. After removing unbound calcein, the CM fish were examined for calcein fluorescent marks on calcified skeletal structures of the head, and dorsal, pectoral, anal, and caudal fins using either Model ML-49 portable UV light or plug in mineral lamp Model UVGL-58 (Ultra-violet Products, Upland, CA) at 365–366 nm wavelength in the dark in the fish facility. The marks were observed daily by placing the UV light source about 5–8 cm above the fish. The percent survival, intensity and duration of the fluorescent marking were determined daily for 45 d.

2.5. Effect of calcein marking on S. iniae susceptibility

Forty tilapia were sham-vaccinated with 100 μL of sterile TSB by ip injection and CM with 500 mg L^{-1} solution for 4 h. Another group of 40 tilapia were sham-vaccinated with TSB and NM. Twenty fish of each group were cohabited in a single aquarium. Two replicate aquaria containing 40 cohabitants/aquarium were prepared (Table 3). The cohabitated fish were maintained in their individual aquaria without disturbance. The fish were cohabitated for 30 d before challenge by ip injection with 100 μL at dose of 15.0×10^7 CFU *S. iniae*/fish.

2.6. Effect of calcein on vaccination and challenge cohabitation protocol

One hundred and sixty tilapia were CM with 500 mg L^{-1} solution for 4 h and sham-vaccinated with TSB by ip injection. One hundred and sixty tilapia were NM and vaccinated with *S. iniae* vaccine by ip injection. Twenty fish from each group were cohabitated in a single aquarium. Eight replicate aquaria containing 40 cohabitants/aquarium were prepared (Table 3).

The infectious isolate was prepared by inoculating 250 mL of TSB in 500 mL culture flask with a thawed aliquot of the frozen isolate. After 24 h at 27 °C incubation, the cultures were adjusted to an optical density of 1.2 at 540 nm using a spectrophotometer to give an adjusted *S. iniae* concentration of 1×10^{10} colony forming units (CFU) mL^{-1} (determined by plate count). Cohabitated fish in replicate aquaria were

Table 2
Calcein concentration, duration of exposure, fish size, number of fish treated, percent (%) survival, percent of fish calcein marked (CM), intensity and duration of calcein marking in bath immersion[a] exposure trials using Nile tilapia

Calcein (mg L^{-1})	Exposure duration (h)	Fish size (g)	Number of fish treated	Duration of CM (d)	Intensity of CM[a]	% of fish CM[a]	% Survival
0	4	15	20	0	absence	0	100
250	4	15	20	25–30	moderate	80	100
500	4	15	20	>45	strong	100	100
250	24	20	20	25–30	moderate	85	100
500	24	20	20	40–45	moderate	90	100
250	48	15	20	23–8	weak	70	100
500	48	15	20	23–28	weak	75	100

[a] Intensity of CM and % of fish CM is visual scoring of marking (absence = not visible, weak = barely visible, moderate = visible, and strong = highly visible) of all fish at time specified in duration of CM.

Table 3
Marking groups reflecting number of Nile tilapia calcein marked (CM) with 500 mg L^{-1} for 4 h or non-marked (NM), vaccination status (sham-vaccinated or vaccinated against *S. iniae*), number of CM and NM tilapia cohabitated, and days cohabited to determine the effect of calcein on *S. iniae* susceptibility or vaccination and challenge cohabitation

Marking groups	Treatment	No. of CM and NM fish	No. of CM and NM fish cohabited	Replicates	Days cohabited
S. iniae susceptibility study					
Calcein marked	Sham-vaccinated (CMSV)	40	20	2	30
Non-marked	Sham-vaccinated (NMSV)	40	20		
S. iniae vaccination/challenge cohabitation study					
Calcein marked	Sham-vaccinated (CMSV)	160	20	8	30
Non-marked	Vaccinated (NMV)	160	20		

challenged by ip injection with 100 μL at doses of 3.5×10^7, 6.8×10^7, 10.0×10^7, or 15.0×10^7 CFU *S. iniae*/fish of adjusted culture.

2.7. Observation and re-isolation protocol

The number of properly CM and NM fish/aquarium was assessed prior to challenge at 31 d and 15 d post-challenge. The challenged fish were monitored for mortality over a 15 d challenge period. Dead fish and survivors were inspected for calcein marks as previously described. To validate *S. iniae* infection, the brain and kidney of dead fish were cultured and identified by previously described techniques. Re-isolation of *S. iniae* was also attempted at 16 d post-protection period in all surviving fish.

2.8. Statistical analysis

Duncan's multiple range tests were used to determine significant differences in mean temperature, dissolved oxygen and pH at 500 mg L^{-1} calcein in 1 L bath solution following immersion of 10 tilapia over 4 h. Mean cumulative mortalities of CMSV and NMSV cohabitants were compared by *t*-tests to determine the effect of marking on fish survival following *S. iniae* challenge. Mean cumulative mortalities of CMSV and NM *S. iniae* vaccinated (NMV) cohabitant tilapia were also compared by *t*-tests at different challenge doses of *S. iniae*. All testing was performed using SAS statistical software, Version 8e [26].

3. Results

3.1. Calcein marking

Examination of the calcified skeletal structures following exposure to 250 and 500 mg/L^{-1} concentration for 4 h revealed that both levels produced detectable fluorescent marking on the tilapia using a portable mineral lamp on the long wave setting (Table 2). The 500 mg L^{-1} level produced the strongest detectable marking with duration of >45 d. The calcified skeletal structures of the head (especially the jaw), dorsal, pectoral, anal, and caudal fins produced the strongest calcein marks.

The results of longer exposures showed that fish exposed for 24 h at 250 and 500 mg L^{-1} levels produced moderate marking. However, the duration of the marking was 25–30 and 40–45 d post-marking at 250 and 500 mg L^{-1}, respectively. Furthermore, 48 h calcein treatments at both levels produced the weakest markings with the shortest duration of 22–28 d. The survival of CM fish was 100% at 250 and 500 mg L^{-1} levels for all time periods evaluated (Table 2). Calcein treated tilapia expressed no abnormal behaviour post-marking. The results of the calcein marking trials indicate that the most suitable calcein concentration and exposure time to produce detectable fluorescent marking of tilapia was 500 mg L^{-1} for 4 h. This level of calcein and exposure time was used in the subsequent cohabitation trials.

3.2. Effect of calcein on S. iniae susceptibility and vaccine and challenge cohabitation model

The cumulative mortality of CMSV tilapia and NMSV tilapia cohabitants was not significantly different ($P = 0.6756$) following *S. iniae* challenge. The results indicate that calcein marking did not affect the susceptibility of fish to *S. iniae* (Table 4). Furthermore, no significant effects of treatment × dose and tank on the percent cumulative mortality were revealed ($P > 0.40$).

Table 4 presents the percent cumulative mortality of CMSV and NMV at 4 colony forming units (CFU) doses/fish at 15 d post-challenge. The percent cumulative mortalities ranged from 50 to 65% in the CMSV cohabitants and 2.5 to 17.5% in the NMV cohabitants at *S. iniae* challenge doses of 3.5 to 15.0×10^7 CFU/fish. Highly significant differences were noted in percent cumulative mortality between NMV and CMSV cohabitants. *S. iniae* vaccination did produce a highly significant ($P = 0.0003$) protective effect. The calcein mark was correctly detected in all CMSV controls at the time of death and in CMSV survivors post-challenge to account 100% of CM fish (Table 4). The failure to assign marked and unmarked fish correctly was not seen.

Table 4
Marking assessment[a] and mean percentage cumulative mortality and survival of calcein marked sham-vaccinated (CMSV) and non-marked sham-vaccinated (NMSV) cohabitants following intraperitoneal (IP) *S. iniae* challenge

Challenge dose	Calcein marked sham-vaccinates (CMSV)				Non-marked sham-vaccinates (NMSV)				Probability
S. iniae CFU/fish	No. fish challenged	Specific losses	% CMSV mortalities	% CMSV survivors	No. fish challenge	Specific losses	% NMSV mortalities	% NMSV survivors	
15.0×10^7	40	26	65	35	40	22	55	45	0.6756

Marking assessment and mean percentage cumulative mortality and survival of CMSV and NM *Streptococcus iniae* vaccinated (NMV) cohabitants at 15 d post-challenge at 4 different doses of *S. iniae* and significant differences between mortality of CMSV and NMSV and CMSV and NMV

Challenge dose	Calcein marked sham-vaccinates (CMSV)				Non-marked *S. iniae* vaccinates (NMV)				Probability
S. iniae CFU/fish	No. fish challenged	Specific losses	% CMSV mortalities	% CMSV survivors	No. fish challenge	Specific losses	% NMV mortalities	% NMV survivors	
3.5×10^7	40	20	50	50	40	1	2.5	97.5	0.0316
6.8×10^7	40	25	63	37	40	7	17.5	82.5	0.0517
10.0×10^7	40	22	55	45	40	6	15.0	85.0	<0.0001
15.0×10^7	40	26	65	35	40	5	12.5	87.5	0.0365
All doses	160	93	58.1	41.9	160	19	11.9	88.1	0.0003

[a] Marking assessment performed on fish at time of death and on all survivors 15 d post-challenge to determine accuracy of marking assignment.

3.3. S. iniae re-isolation from dead and surviving fish

All dead fish were cultured positive for *S. iniae* following challenge. Attempts to re-isolate *S. iniae* from either the vaccinated or non-vaccinated fish at the end of the 15 d protection period were negative. However, signs of *S. iniae* infection that include eye opacity, body curvature, bottom location, erratic swimming, slow acceptance of food, or lethargic behaviour were present in non-vaccinated CM tilapia.

4. Discussion

The results show that calcein marking is a non-lethal, non-injurious, and cost-effective technique to produce marks in calcified skeletal structures of 15–20 g Nile tilapia. The marks can be successfully detected with commercially available handheld UV lamps. It was found that a calcein concentration of 500 mg L^{-1} for 4 h produced detectable marking of tilapia for longer than 45 d without undue stress or mortality (Table 4). The calcein marking can also be detected using a sample of excised caudal fin viewed under a fluorescent microscope (results not shown). Furthermore, calcein marking did not promote increased *S. iniae* susceptibility or horizontal transmission of infection between the cohabitated fish populations. Mortality of CMSV and NMSV groups subjected to the same vaccination and challenge procedure was not significantly different. The normal behaviour and feeding activity of fish marked with calcein indicated that this technique was suitable for distinguishing between cohabitants in the vaccination and challenge cohabitation model described in this study.

In the present study, calcein marking of sham-vaccinates did not affect the vaccine and challenge cohabitation trial. The *S. iniae* vaccine produced 95% reduction in mortality for cohabitated fish challenged with *S. iniae* at concentration of 3.5×10^7 CFU/fish. This is in agreement with earlier work, where the *S. iniae* vaccine used in the present study was previously found to reduce mortality by 91.3% in tilapia weighing 25–100 g [21] following ip injection.

Nordmo [1,27] reviewed the strengths and weakness of different challenge methods used to determine the efficacy of fish vaccines. Immersion and cohabitation routes were considered the challenge methods that most closely mimic natural routes of infection [28]. However, Nordmo [1,27] indicated that ip challenge was a reproducible and reliable means for the determination of vaccine efficacy. In addition, the ip route ensures that each individual fish receives a uniform challenge; however, it is generally considered to be the least natural route of infection, unless the fish are subject to skin puncture by infected fish [1,27]. Nordmo [1] also suggested that ip injection is acceptable where it is the primary cause of mortality (i.e., no secondary peaks of mortality caused by waterborne infection). In previous vaccine studies, no secondary mortalities caused by waterborne infections from the inoculated challenged fish were observed [21,22]. Cohabitation challenge of tilapia may produce sufficient mortality, but may be subjected to unacceptable variability in rates of mortality between tanks and experimental trials. Finally, for an immersion and cohabitation challenge method to work, the pathogen must be able to induct infection via water or other media present in the test system [1]. In a previous study, immersion exposure of tilapia to *S. iniae* failed to cause sufficient mortalities (27%) in order to adequately evaluate *S. iniae* vaccine efficacy [24]. Thus, we chose to challenge the CMSV and NMSV cohabitants and CMSV and NMV cohabitants by ip injection in this study.

Jarp and Tverdral [29] indicated that in an experimental vaccine trial the design should ensure that the groups to be compared are equal in all aspects except for the factor to be assessed (vaccine). In addition, these authors suggested that the fish should be considered the experimental unit rather than aquaria. To meet these design requirements, we developed and evaluated our vaccination and challenge cohabitation model using CM fish. The results show that the cumulative mortality of NMSV and CMSV was significantly greater than those of NM *S. iniae* vaccinates (Table 4) indicating that calcein did not have an

effect on disease susceptibility. Furthermore, no significant effect was noted for tank, tank versus treatment, challenge dosage, and calcein marking.

The development of this model for evaluation of fish vaccines should enable researchers to better test and evaluate various fish vaccines. This model also offers the statistical advantage of using fish as the experimental unit under the same experimental conditions. This model may also better permit the evaluation of different vaccine formulations, routes of vaccine administration, and duration of protection. Previously, Erdal and Reitan, [30] evaluated the immune response and protective immunity after vaccination of Atlantic salmon against furunculosis using a cohabitation challenge model. However, these investigators employed adipose fin clips to distinguish the vaccinates from control fish. Furthermore, in their study, the vaccinates and controls were kept in separate replicate tanks and only cohabitated at the time challenge with *Aeromonas salmonicida* and for the duration of the protection period. In this proposed model, the vaccinates and control fish are cohabitated throughout the entire evaluation period using a marking technique that does not promote the risks of horizontal transmission of infection or increase disease susceptibility. It is likely that this model will be useful to compare different vaccine lots, competitive vaccines, and vaccine efficacy under varied conditions. Further experiments are planned to compare results obtained for vaccine efficacy from laboratory and field studies. In addition, information obtained from immersion routes of vaccination and challenge with the appropriate pathogen will be assessed in further evaluation of this model.

Acknowledgements

We thank Crystal Braden, Dave Carpenter, and Curtis Day for their excellent and invaluable help in the laboratory and experimental fish facilities. We extend special thanks to Dr. John Grizzle, Department of Fisheries and Allied Aquaculture, Auburn University, and Dr. Victor Panangala and Lisa Biggar (USDA, ARS, Aquatic Animal Health Research Laboratory) for their review and helpful editorial suggestions. Our gratitude to Deb Boykin, USDA, ARS, Mid South Area Office, Mississippi and Dr. Dehai Xu, USDA, ARS, Aquatic Animal Health Research Laboratory, Alabama for valuable help during the statistical analysis.

References

[1] Nordmo R. Strengths and weaknesses of different challenge methods. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Fish vaccinology, vol. 90. Basel, Switzerland: Karger Publishers; 1997. p. 303–9.
[2] Raynard RS, Snow M, Bruno DW. Experimental infection models and susceptibility of Atlantic salmon *Salmo salar* to a Scottish isolate of infectious salmon anemia virus. Diseases of Aquatic Organisms 2001;47:169–74.
[3] Faragher RA, Gordon GNG. Comparative exploitation by recreational anglers of brown trout, *Salmo trutta* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Lake Eucumbene, New South Wales. Australian Journal of Marine and Freshwater Research 1992;43:835–45.
[4] Hughes TC, Josephson DC, Krueger CC, Sullivan PJ. Comparison of large and small implant tags: retention and readability in hatchery brook trout. North American Journal of Aquaculture 2000;62:273–8.
[5] Courtney DL, Mortensen JA, Orsi JA, Munk KM. Origin of juvenile Pacific salmon recovered from coastal Southeastern Alaska identified by otolith thermal marks and coded wire tags. Fisheries Research 2000;46:267–78.
[6] Mohler JW. Immersion of larval Atlantic salmon in calcein solutions to induce a non-lethally detectable mark. North American Journal of Fisheries Management 1997;17:751–6.
[7] Elliott DG, Pascho RJ. Evidence that coded-wire-tagging procedures can enhance transmission of *Renibacterium salmoninarum* in Chinook salmon. Journal of Aquatic Animal Health 2001;13:181–93.
[8] Leips J, Baril CT, Rodd FH, Reznick DN, Bashey F, Visser GJ, et al. The suitability of calcein to mark poeciliid fish and a new method of detection. Transactions of the American Fisheries Society 2001;130:501–7.
[9] Du SJ, Frenkel V, Kindschi G, Zohar Y. Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. Developmental Biology 2001;238:239–46.

[10] Reznick DN, Bulter MJ, Rodd FH, Ross P. Life history evolution in guppies: VI. Differential mortality as a mechanism for natural selection. Evolution 1996;50:1651–60.
[11] Rodd FH, Reznick DH. Life history evolution in guppies: III. The impact of pawn predation on guppy life histories. Oikes 1991;62:13–9.
[12] Bumguardner BW, King TL. Toxicity of oxytetracycline and calcein to juvenile striped bass. Transactions of the American Fisheries Society 1996;125:143–5.
[13] Thomas SA, Holt LM, Arnold CR. Chemical marking techniques for larval and juvenile red drum (*Sciaenops ocellatus*) otoliths using different fluorescent markers. In: Secor DH, Dean JM, Campana SE, editors. Recent developments in fish otolith research. First International symposium of fish otoliths: research and application. Hilton head, SC, January, 1993. Columbia, SC: University of South Carolina Press; 1995. p. 703–11.
[14] Wilson CA, Beckman DW, Dean JM. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. Transactions of the American Fisheries Society 1987;11:668–70.
[15] Beckman DW, Wilson CA, Lorica F, Dean JM. Variability in incorporation of calcein as a fluorescent marker in fish otoliths. American Fisheries Society Symposium 1990;7:547–9.
[16] Mohler JW, Millard JM, Fletcher JW. Predation by captive wild brook trout on calcein-marked versus nonmarked Atlantic salmon fry. North American Journal of Fisheries Management 2002;22:223–8.
[17] Frenkel V, Kindschi G, Zohar Y. Noninvasive, mass marking of fish by immersion in calcein: evaluation of fish size and ultrasound exposure on mark endurance. Aquaculture 2002;214:169–83.
[18] Hales Jr LS, Hurley DH. Validation of daily increment formation in the otoliths of juvenile silver perch, *Bairdiella chrysoura*. Estuaries 1991;14:199–206.
[19] Monaghan Jr JP. Comparison of calcein and tetracycline as chemical markers in Summer flounder. Trans American Fisheries Society 1993;122:298–301.
[20] Mohler JW. Producing fluorescent marks on Atlantic salmon fin rays and scales with calcein via osmotic induction. North American Journal of Fisheries Management 2003;23:1108–13.
[21] Klesius PH, Shoemaker CA, Evans JJ. Efficacy of a killed *Streptococcus iniae* vaccine in tilapia (*Oreochromis niloticus*). Bulletin of the European Association of Fish Pathologists 1999;19:39–41.
[22] Klesius PH, Shoemaker CA, Evans JJ. Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*). Aquaculture 2000;188:237–46.
[23] Shoemaker CA, Klesius PH. Streptococcal disease problems and control – a review. In: Fitzsimmons K, editor. Tilapia aquaculture proceedings from the fourth international symposium on tilapia in aquaculture. Ithaca, NY: Northeast Regional Agricultural Engineering Service Cooperative Extension; 1997. p. 671–82.
[24] Shoemaker CA, Evans JJ, Klesius PH. Density and dose: factor's affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*). Aquaculture 2000;188:229–35.
[25] Klesius PH, Shoemaker CA, Evans JJ. *Streptococcus iniae* vaccine. U.S. patent No. 6,379,677B1; 2002.
[26] SAS Institute Inc. 4th ed. In: SAT/STAT user's guide, Version 6, vol. 2 Cary, NC: SAS Institute Inc.; 1989.
[27] Nordmo R, Ramstad A. Comparison of different challenge methods to evaluate the efficiency of furunculosis vaccines in Atlantic salmon (*Salmo salar* L.). Journal of Fish Diseases 1997;20:119–26.
[28] Midtlyng PJ, Reitan LJ, Lillehaug A, Ramstad A. Protection, immune responses and side effects in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures. Fish and Shellfish Immunology 1996;6:599–613.
[29] Jarp J, Tverdal A. Statistical aspects of fish vaccination trials. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Fish vaccinology, vol. 90. Basel, Switzerland: Karger Publishers; 1997. p. 311–20.
[30] Erdal JI, Reitan LJ. Immune response and protective immunity after vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1992;2:99–108.